Advances in Bioresearch
Adv. Biores., Vol 10 (4) July 2019: 51-62
©2019 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.10.4.5162

Advances in Bioresearch

ORIGINAL ARTICLE

Isolation, Identification and Optimization of Protease Producing Bacteria *Bacillus gaemokensis* from Poultry Soil

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ABSTRACT
The present study aimed to isolate and identify the bacterial strain producing protease enzyme and optimize the culture conditions for its maximum degrading activity. Soil collected from poultry, serially diluted and three strains namely SAL-S1, SAL-S2 and SAL-S3 which showed potent protease activity on skim milk agar plates were isolated. Further screening was performed by protease assay, and identified that the strain SAL-S2 was most potential producer of protease among the other strains. Morphology, colony assay and biochemical test were performed for the selected strain to determine the specific type of bacteria. The result showed that the isolate belongs to Bacillus genus. Further,16SrRNA sequencing was carried out to identify the specific species of Bacillus and the resulting bacterial strain isolate was found to be Bacillus gaemokensis and the gene bank accession number was KY938881. After identification, the culture conditions required for maximum protease production were optimized.
Key words: *Protease activity, Poultry soil, SAL-S2, Bacillus gaemokensis, Genbank number: KY938881.*

Received 04.01.2019 Revised 28.04.2019 Accepted 16.02.2019

How to cite this article:
Salamun DE, A Shetty, M Shah, S S Patel, Sneha J. Isolation, Identification and Optimization of Protease Producing Bacteria *Bacillus gaemokensis* from Poultry Soil. Adv. Biores., Vol 10 [4] July 2019.51-62.

INTRODUCTION

Enzymes are proteinaceous in nature that perform the catalyzation of several biochemical steps inside the body. There are various source for isolation of enzymes in nature however microbial enzymes have a vast use in industry and in medical field [1]. Microbial enzymes are preferred over plant and animal enzymes because they can be cultured on a large scale and microbial enzymes are relatively more stable and active. Proteases are hydrolytic enzymes that act on proteins and degrade them into peptides or amino acids [2]. They cleave the peptide bond that link the amino acids in a protein substrate using a water molecule. Proteases are ubiquitous in nature and are found in plants, animals, and microorganisms. Plants as a source of protease has a limitation production time. Animal proteases include trypsin, rennin, chymotrypsin etc. The disadvantage of animal as a source of protease is the ethical issue involved in it. The advantages of using microorganism as a source of proteases are: Broad biochemical diversity, Amenability to genetic manipulation and highly stable and active enzyme [3].

The microorganisms that produce proteases are Bacteria, Fungi and some Viruses. Among Bacteria, the organisms belonging to the genus *Bacillus* are the most potent producers of proteases [4]. With the increase in the production of poultry meat, huge amount of wastes are produced during the slaughter of chicken. There are several problems caused by the poultry waste to the environment like the odour, flies, soil pollution, the contaminated water can cause eutrophication etc.The chicken feathers are often buried, incinerated and less frequently recycled into feeds for animals [5]. Apart from feather, hair which are the by product from tanneries during haircut process, wool form sheep are also keratin rich wastes that generate environmental problems [6]. Conversion of keratin rich material into low-nutritive feed by chemical and thermal hydrolysis poses serious threat to environment because of the use of toxic reagents and heat. At present, bioconversion of insoluble keratin into more soluble form using keratinolytic microorganism provides an eco-friendly, cost effective alternative[7].Therefore the aim the study was to

isolate protease producing bacteria from poultry soil, to determine the various factors that influences the production of enzyme activity and to identify the proteolytic potential of the isolated bacterial strain from industrial waste.

MATERIA AND METHODS

Source of sample

Soil samples were collected from a poultry near a village in Tumkur district. Soil was collected from the surface.

Isolation of protease producing bacteria

1g of soil sample was accurately weighed and added to 9ml of sterile distilled water. This gave 10^{-1} soil sample and it was further diluted to 10^{-2} 10^{-3}, 10-4, 10^{-5} dilutions. 0.1ml of each diluted samples were inoculated onto the skim milk agar plates. The plates were incubated 37°C for 24 hours. The colonies that grew on the skim milk agar plates were positive for the production of protease[8].

Selection of the dominant protease producing colony and its confirmatory test

From the spread plates of skim milk agar, 1 dominant colony was selected from each dilution. A quadrant streak inoculation was done on nutrient agar plates for isolation of pure culture. The plates were incubated at 37°C for 24 hours. Protease production was confirmed by casein hydrolysis on skim milk agar plates. A single colony was chosen from the fourth quadrant and was inoculated on to the skim milk agar plates as a single streak. The plates were incubated at 37°C for 48hours for the production of enzyme. After 48 hours 5ml of 10% $HgCl_2$ was poured into the petriplates. The colonies showing the highest zone of clearance confirmed the proteaseproduction.

The positive isolates were further screened for better production of enzyme by assaying the protease activity in liquid culture using casein as a substrate at 37°C. The isolate which showed maximum activity was selected for further studies[8].

Protease assay

Protease estimation was done using Lowry's method, the tyrosine standard Graph was constructed by pipetting 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml of the tyrosine stock solution (0.5µg/ml) in different tubes. The absorbance of the tubes were read at 660nm against a suitable blank [9-10].

Identification and characterization of isolated bacteria

The isolate was identified and characterized based on cellular morphology and biochemical test. Further, 16SrRNA sequencing was performed to confirm the identity of the isolate. For morphological identification, Gram staining and endospore staining were performed followed by colony characterization. For biochemical characterization, Indole production test, MR-VP test, Citrate utilization test, Catalase test and Starch hydrolysis tests were performed [11].

Estimation of total protein content

The total protein content of the identified culture were determined by Lowry's method. The protein standard used was tyrosine 0.5mg/ml. Different volumes of the stock solution in ml-0.2,0.4,0.6,0.8,and 1 was pipetted into different test tubes. The change in the color of the test and standard tubes were read against blank at 660 nm. The total protein content of the sample was determined using a standard curve [12].

Optimization of parameters for protease production

The media used for optimization was Mineral Salt Media (MSM) containing g/l of KH_2PO_4- 0.42; K_2HPO_4- 0.375; $(NH_4)_2SO_4$ - 0.244; NaCl- 0.015; $CaCl_2$. $2H_2O$ - 0.015; $MgSO_4.7H_2O$ - 0.05; and $FeCl_3.6H_2O$ - 0.054 and pH 7.0

Carbon supplements

The MSM was supplemented with different carbon sources such as 1% (w/v) - lactose, glucose, maltose and sucrose. The tubes were incubated at 37^0C for 48 hours. After incubation, protease activity assay was carried out to determine the most potential carbon substrate that induces the maximum protease enzyme production [13].

Nitrogen supplements

The MSM was supplemented with different nitrogen sources such as 1 %(w/v) - Peptone, beef extract, yeast extract and sodium nitrate. The tubes were incubated at 37°C for 48 hours. After incubation, protease activity assay was carried out to determine the most potential nitrogen substrate that induces the maximum protease enzyme production[13].

pH

The pH of the medium was adjusted using 1N HCl or 1N NaOH. The tubes were incubated at 37^0C for 48 hours. After incubation, protease activity assay was carried out to determine the optimum pH that induces the maximum protease enzyme production [13].

Temperature

The inoculated tubes were incubated at different temperatures 30°C, 37°C and 45°C for 48 hours. After incubation, protease activity assay was carried out to determine the optimum temperature that induces the maximum protease enzyme production [13].

Determination of proteolytic potential of the isolate on different keratinous substrates

The keratin substrates were first washed under tap water, followed by washing under distilled water. They were then degreased using methanol: chloroform(1:1)solution. The identified isolate was grown in the mineral salt medium supplemented with different keratin containing substrates such as 1% (w/v) bristles of chicken feather, Goat hair, Barb of the feather and Human hair. The tubes were incubated at 37°C for 5 days. After incubation, protease activity assay was carried out to determine most suitable keratin containing substrate that induces the maximum protease enzyme production [14,15].

RESULTS

Isolation of protease producing bacteria

The colonies of bacteria that grew on the skim milk agar plates, utilized casein in the medium and were positive for the production of protease.

From the skim milk agar plates, three dominant colonies named as SAL-S1, SAL-S2 and SAL- S3 colonies were taken and single streaked onto the fresh skim milk agar plates to screen for the most dominant protease producer (Fig 1a, 1b, 1c). After 48 hours of incubation at 37°C, there was abundant growth of the bacterial isolates. By the addition of $HgCl_2$, the zone of clearance was measured in mm as shown in Table 1.

Among the three isolates, SAL-S1 and SAL-S2 strains showed large zone of clearance and were chosen for further studies. These two strains were further screened for better production of enzymes, by assaying the enzyme activity in liquid culture, using casein as the substrate at 37□ for 48 hours. Further experiments were carried out with SAL-S2 strain as it showed higher enzyme activity - 0.18 U/mL, compared to SAL-S1 with an enzyme activity of 0.058 U/ml (Graph 1).

Identification and characterization of isolated bacteria

Grams' staining and endospore staining were performed for morphological identification of the organism followed by colony characterization. For biochemical characterization, MR-VP test, Indole production test, Citrate utilization test ,Catalase test, Starch hydrolysis test were performed.

Molecular identification

Partial 16S rRNA Sequencing

The molecular approaches for detecting and clarifying the proteolytic bacterial species was performed by the partial 16srRNA sequencing, which rely on the amplification and analysis of 16srRNA. The apparent size of PCR amplicon was ~1.5kb.

The bacterial isolate was identified using molecular identification by partial 16SrRNA sequencing and the obtained 850bp partial 16S rRNA nucleotide sequence was compared with available 16S rRNA sequences in NCBI using BLASTn. The proteolytic bacteria has been enrolled into a cluster containing Bacillus sp. and was found to be closely related to *Bacillus gaemokensis* strain BL3-6 with 99% sequence similarity (Fig. 3). Hence, it was designated as *Bacillus gaemokensis.* The nucleotide sequence was submitted and was provided with a Gen bank accession number: KY938881. Thus identified, the strain belonged to *Bacillus species*. The Queried strained is played 99% similarity with the closest relative *Bacillus gaemokensis* strain BL3-6*.*

Estimation of total protein content Lowry's method

The total protein content of the test sample was estimated using Lowry's method and the protein content was found to be 400µg/ml. (Graph 2).

Optimization of parameters for protease production

Effect of different carbon source

Among the various carbon sources used, protease production was highest in the medium containing lactose (0.405 U/ml), followed by glucose (0.309 U/ml) (Graph 3). Use of maltose and sucrose as the carbon sources yielded protease production which was almost similar, 0.28 and 0.27 U/ml respectively. It was observed that protease production was found to be maximum with lactose (140 U/ml) [16].

Effect of different nitrogen source

Various nitrogen sources were investigated for optimizing the protease production. Medium containing Beef extract (0.3 U/ml) showed highest protease productions followed by peptone (0.2 U/ml) and yeast extract(0.1U/ml),whereas sodium nitrate showed the least enzyme activity of 0.057U/ml(Graph4). The observations from this findings infers that organic source was found to be effective ingredients for protease productions than inorganic source. No previous literature report to support the effectiveness of Nitrogen source in protease production from the newly isolated strain *Bacillus gaemokensis* [17, 18].

Effect of different pH

The isolate *Bacillus gaemokensis* was tested for the enzyme production at various pH- 4.0, 6.0, 7.0 and 9.0. Maximum protease production was observed at pH- 7.0 (0.296 U/ml) (Graph 5). The protease production increased with the increase in pH from acidic to neutral and decreased at alkaline pH [19, 20, 21, 22, 23].

Effect of different temperature

Temperature is the most influencing factor that affects the enzyme activity. Maximum enzyme activity was achieved at optimum temperature which varies from species to species. The bacterial isolate *Bacillus gaemokensis* was incubated at different temperatures like 30℃, 37 ℃ and 45℃. Maximum protease production was observed at 37℃ showing protease activity of 0.423U/ml.(Graph6) [19, 22, 24]

Application of proteolytic potential of the isolate on different keratinous substrates

When different keratin containing substrates were used, the maximum production of enzyme [3.65 U/ml]occurred when feather bristles was used as a sole source of Carbon and Nitrogen(Fig-4a, 4b, 4c and 4d). Feather Barb as a substrate showed an enzyme production of 2.82 U/ml, followed by goat hair 1.15 U/ml (Graph 7). Human Hair as a substrate showed least protease enzyme production of 0.01U/ml making it a less favorable substrate. This might be due to higher disulphide bridges and increased cystine content present in human hair compared (15.5 %) compared to other keratin substrates feather (6.8 %) and Goat hair(8.9%) making the proteolytic enzyme difficult to act on them[6, 25].

Fig-1a: SAL-S1 showing zone of clearance

Fig-1b: SAL-S2 showing zone of clearance

Fig-1b: SAL-S2 showing zone of clearance

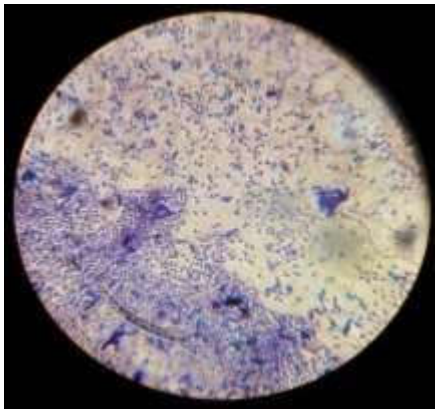


Fig-2: Gram staining showing Gram positive *Bacilli*

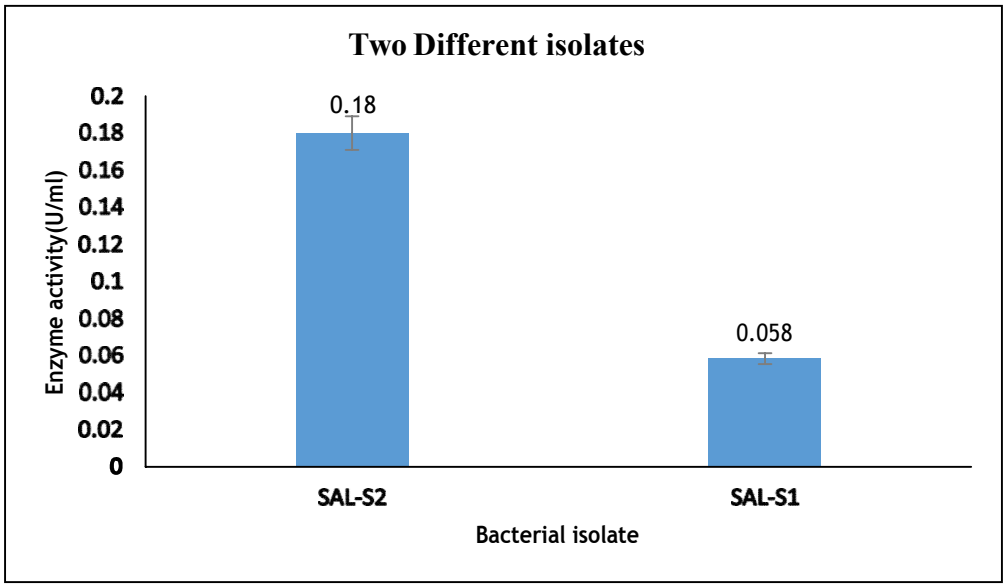
Figure- 3:Phylogenetic tree showing the relationship with *Bacillus gaemokensis* strain BL3-6 and related *Bacillus sp.* from NCBI database of partial 16S rRNA sequences

Fig-4a: Submerged fermentation using feather bristle as a substrate

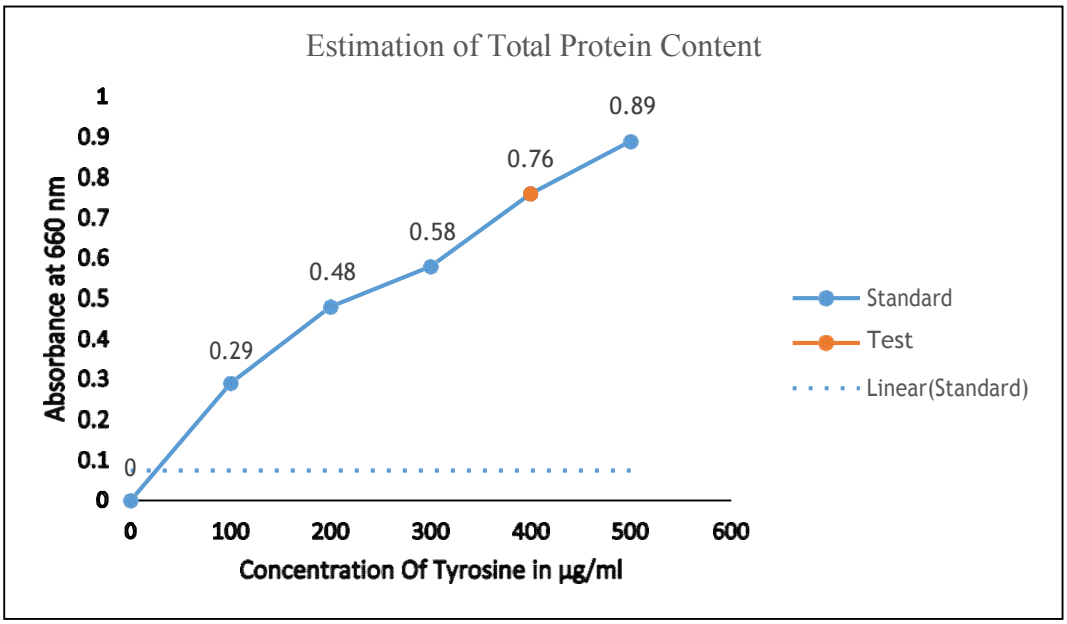
Fig-4b:Submerged fermentation using barb of feather as a substrate

Fig-4c: Submerged fermentation using Goat hair as a substrate

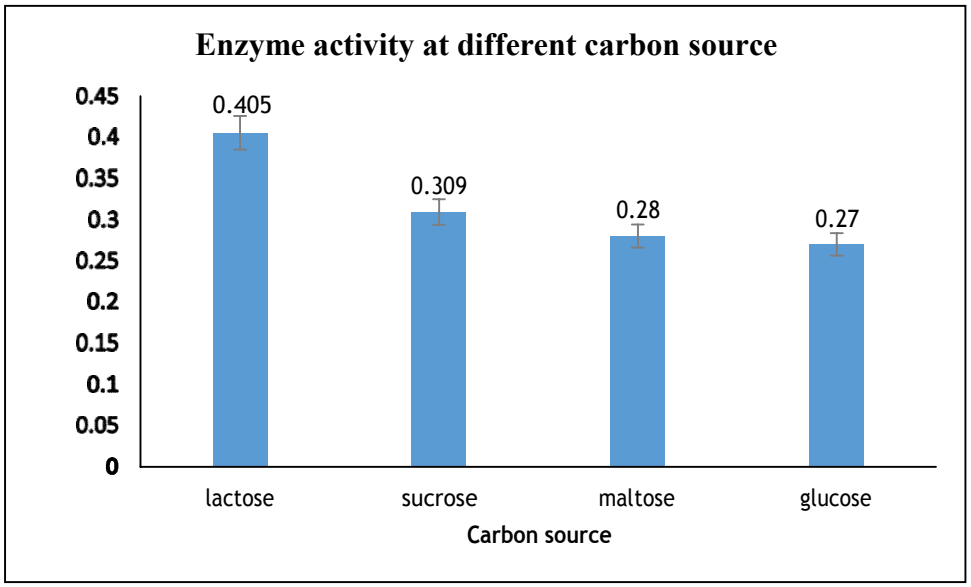
Fig-4d: Submerged fermentation using human hair as a substrate



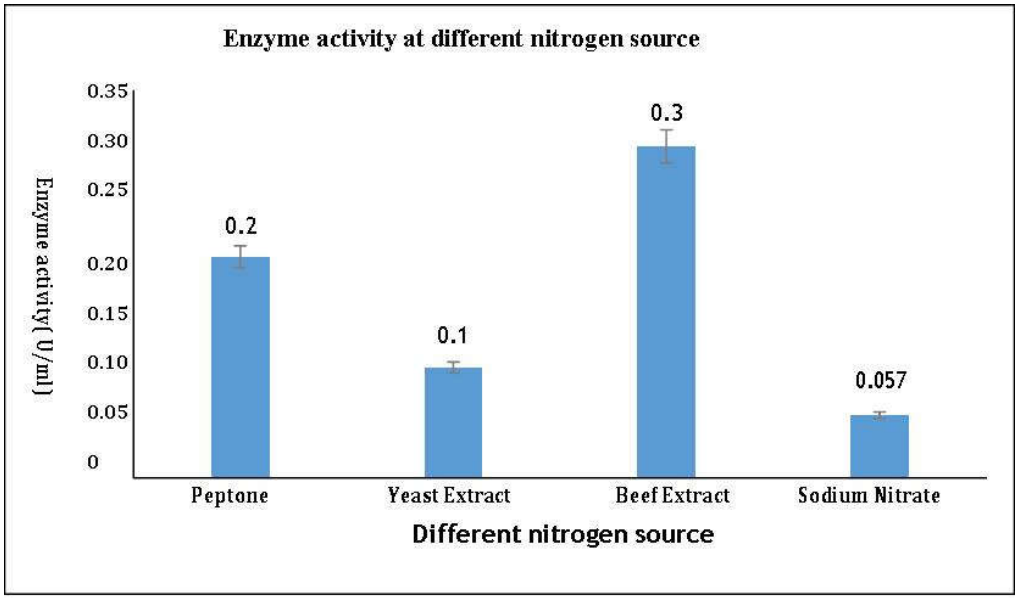
Graph-1: Enzyme Activities of the two isolates



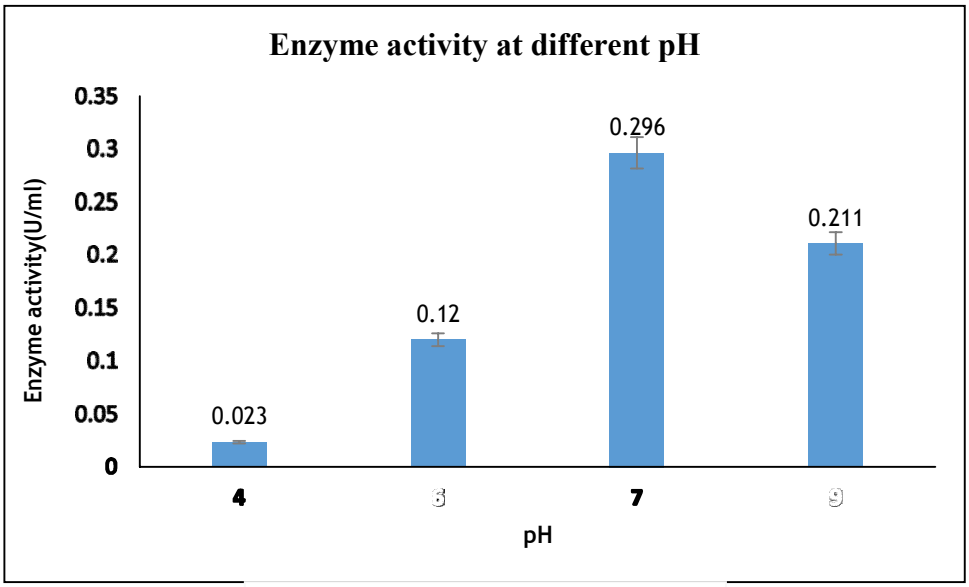
Graph-2: Estimation of total protein content by Lowry's method



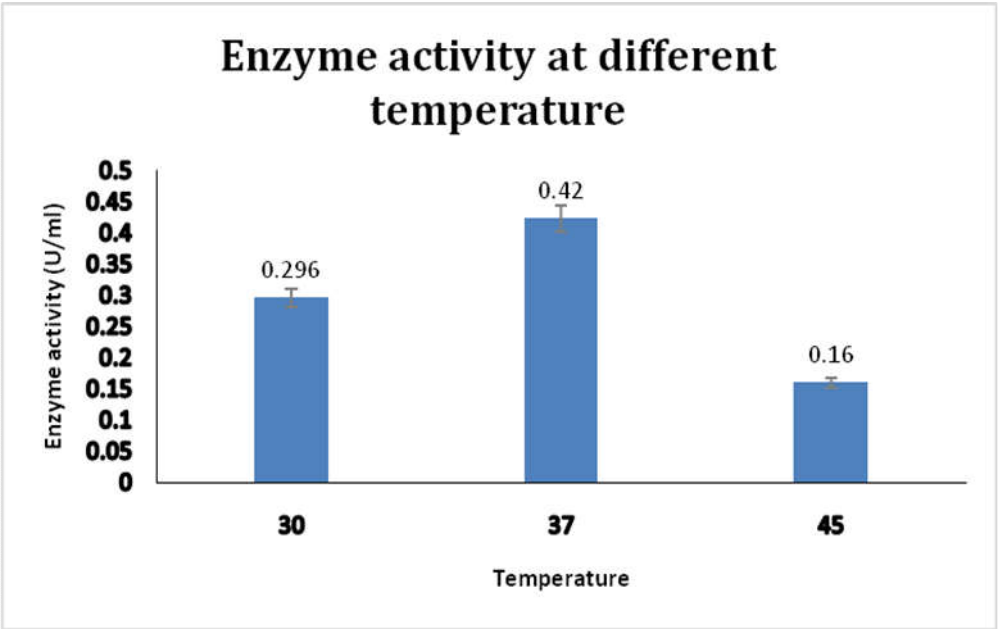
Graph-3: Enzyme activity at different carbon source



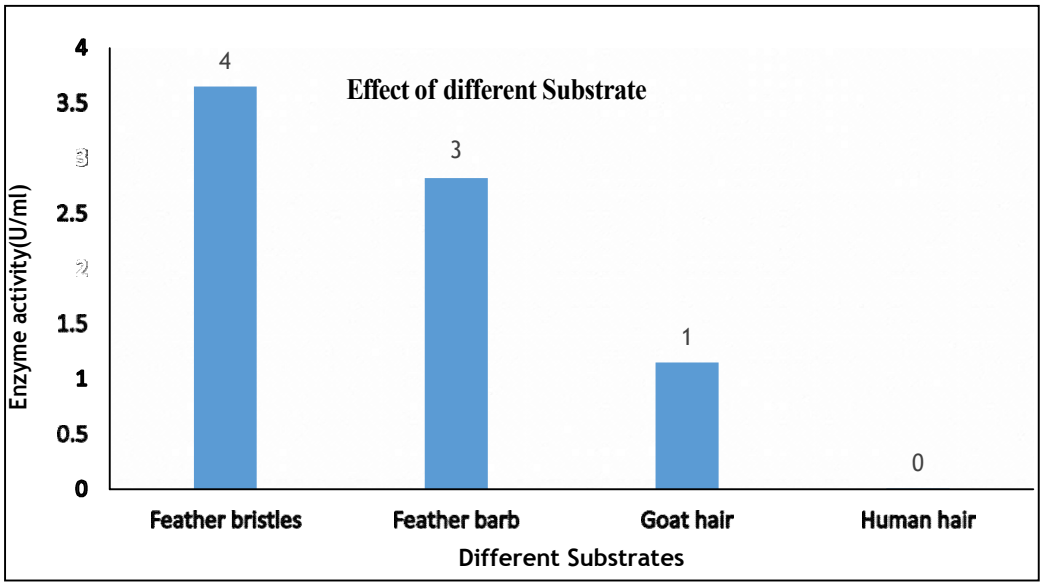
Graph-4: Enzyme activity at different nitrogen sources



Graph-5: Enzyme activity at different pH



Graph-6: Enzyme activity at different temperature



Graph-7: Enzyme activity at different substrates

Table-1: The Measurement of Zone of Clearance

ISOLATE	ZONE OF CLEARANCE
SAL – S1	28 mm
SAL – S2	30 mm
SAL – S3	08 mm

Table-2: Colony characteristics of SAL-S2

CHARACTERISTICS	RESULT
Shape	Round
Elevation	Flat
Margin	Entire
Surface	Smooth
Color	White
Degree of growth	Moderate

Table-3: Morphological characteristics of SAL-S2

GRAM CHARACTER	POSITIVE
ENDOSPORE STAINING	POSITIVE

Table -4: Biochemical Characteristics

INDOLE TEST	NEGATIVE
METHYL RED TEST	POSITIVE
VOGES PROSKAUER TEST	NEGATIVE
CITRATE UTILIZATION TEST	POSITIVE
STARCH HYDROLYSIS TEST	POSITIVE
CATALASE TEST	POSITIVE

DISCUSSION

The present study aimed to isolate and identify the bacterial strain producing protease enzyme and optimize the culture conditions for its maximum degrading activity. The soil wastes generated from the slaughter house like bedding materials, feathers, dead embryos, shells and blood could be degraded by

this bacterial species, since huge amount of poultry and industrial wastes generated causes odour, soil pollution and the contaminated water can cause eutrophication as well.

Thesamplesource,soilwascollectedfromthepoultrynearbyTumkurdistrict.Initiallythreestrainsnamely SAL-S1, SAL-S2 and SAL-S3 which showed potent protease activity on skim milk agar plates were isolated. Further screening was performed by protease assay, and identified that the strain SAL-S2 was the most potential producer of protease among the other strains. Therefore, further optimization for maximum enzyme production was carried out throughout the experiment using SAL-S2 strain.

Morphology, colony assay and biochemical characterization were performed for the selected strain to determine the specific type of bacteria. The results showed that the isolate belongs to the *Bacillus* genus. Further, 16S rRNA partial sequencing was carried out to identify the specific species of *Bacillus* and the resulting bacterial strain isolate was found to be *Bacillus gaemokensis* and the Gene bank access ion no was KY938881.

After identification, the cultural conditions required for maximum protease production by the organism were optimized. In the present study, it was observed that the *Bacillus gaemokensis* SALS utilized lactose as the carbon source and producing acid during fermentation and showed maximum protease production. Our findings was in agreement with the observation of Vidushi Khajuria *et al*., [16], who observed and reported that among various carbon sources used, protease production was found to be maximum with lactose (140 U/ml) [18].

In nitrogen source – Beef extract (0.3 U/ ml) showed the highest protease activity. Beef extract is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals and some vitamins, providing an undefined source of nutrients which supports good growth of diverse microorganisms. A combination of yeast extract and peptone was found to be the optimum nitrogen source for protease production from the findings of Gopal K Joshi *et al*., [17]. No previous literature report to support the effectiveness of Nitrogen source in protease production from the newly isolated strain *Bacillus gaemokensis* SALS. The observations from this findings infers that organic source was found to be effective ingredients for protease productions than inorganic source and shows the versatility of the bacterial species utilizing a wide range of compounds. pH of the media plays an important role in inducing the production of protease. The isolate *Bacillus gaemokensis* SALS was tested for the enzyme production at various pH-4.0,6.0,7.0and9.0 and maximum protease production was observed at pH-7.0(0.296U/ml). The bacterial isolate *Bacillus gaemokensis* SALS was incubated at different temperatures like 30^0C, 37^0C and 45^0C . Maximum protease production was observed at 37^0C showing protease activityof0.422U/ml.

The strain *Bacillus gaemokensis* SALS was tested for its ability to degrade various keratin containing substrates by submerged fermentation. Maximum protease enzyme production was noted when bristles of white chicken feather was used as a sole source of carbon and nitrogen. This is due to rich cysteine content (15%) present in human hair compared to feather and goat hair containing (2.2 % & 3 %) respectively.

ACKNOWLEDGMENT

The authors are grateful to the management of Jain (Deemed-to-be University) for providing required facilities for carrying out the research work.

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